Myeloperoxidase in Human Intracranial Aneurysms
Preliminary Evidence

Matthew J. Gounis, PhD; Srinivasan Vedantham, PhD; John P. Weaver, MD; Ajit S. Puri, MD; Christopher S. Brooks, PAC; Ajay K. Wakhloo, MD, PhD; Alexei A. Bogdanov Jr, PhD, DSc

Background and Purpose—Noninvasive imaging identifying a predictive biomarker of the bleeding risk of unruptured intracranial aneurysms (UIAs) is needed. We investigated a potential biomarker of UIA instability, myeloperoxidase, in human aneurysm tissue.

Methods—Human brain aneurysms were harvested after clipping and were histologically and biochemically evaluated for the presence of myeloperoxidase. Of the tissue collected, 3 were from ruptured aneurysms and 20 were from UIAs. For each UIA, its 5-year aneurysm rupture risk was determined using the Population, Hypertension, Age, Size of Aneurysm, Earlier Subarachnoid Hemorrhage From Another Aneurysm and Site of Aneurysm (PHASES) model.

Results—All ruptured aneurysms were myeloperoxidase positive. Of the UIAs, half were myeloperoxidase positive. The median 5-year aneurysm rupture risk was higher for myeloperoxidase-positive UIA (2.28%) than myeloperoxidase-negative UIA (0.69%), and the distributions were statistically different (P<0.005, Wilcoxon–Mann–Whitney test). The likelihood for myeloperoxidase-positive UIA was significantly associated (P=0.031) with aneurysm rupture risk (odds ratio, 4.79; 95% confidence limits, 1.15–19.96).

Conclusions—Myeloperoxidase is associated with PHASES estimated risk of aneurysm rupture and may potentially be used as an imaging biomarker of aneurysm instability. (Stroke. 2014;45:1474-1477.)

Key Words: biomarker ■ inflammation ■ intracranial aneurysm

Inflammation of the cerebral aneurysm wall has been identified as an important characteristic predictive of aneurysm progression to rupture (reviewed in Tulamo et al1). Numerous unruptured intracranial aneurysm (UIA) pathology studies have shown the presence of neutrophil-derived enzymes (eg, elastase, neutrophil gelatinase-associated lipocalin–matrix metalloproteinases-9 complexes) in aneurysms. These enzymes promote medial matrix degradation and are associated with vascular instability.2,3 Myeloperoxidase is a secretable oxidoreductase of azurophilic granules of polymorphonuclear cells (primarily neutrophils). In the presence of H2O2, which is generated by neutrophilic respiratory burst, secreted myeloperoxidase produces chlorinating bactericidal species such as hypochlorous acid. In addition to a well-known role in host defense system against microorganisms, myeloperoxidase has been recently implicated in the initiation and destabilization of atherosclerotic plaques (reviewed in Nicholls and Hazen4).

Previously, we demonstrated that the enzymatic activity of myeloperoxidase can be used as a highly selective and sensitive target for inflammation imaging using clinical modalities (MRI, single-photon emission computed tomography).5-7 Rabbit model studies validated the use of a gadolinium (III)-chelate myeloperoxidase activity–sensing MRI agent as a probe for the detection and localization of inflammation in a saccular aneurysm model.8 The local accumulation of the imaging agent correlated with the presence of myeloperoxidase activity at the site of aneurysm inflammation. The resulting magnetic resonance signal intensity changes were quantifiable at clinically relevant magnetic resonance field strength and gadolinium (III) dosages.8

Although these results are promising, there is no direct evidence of the presence of myeloperoxidase in human aneurysm tissues. The present study was designed to investigate the association between myeloperoxidase in human aneurysms with known risk factors for rupture.

Materials and Methods
Myeloperoxidase Levels in Human Aneurysm Tissue and Histology
Human aneurysm tissue was resected during microsurgical clipping of brain aneurysms under an institutional review board–approved...
protocol. After secure clip placement, the aneurysm dome was resected, collected in sterile saline, embedded in tissue freezing medium (Triangle Biomedical Sciences, Durham, NC) and snap-frozen in liquid nitrogen. The specimens were stored at −80°C until histological processing (see protocols in online-only Data Supplement). When the tissue specimen was sufficiently large, ≈50% of the tissue was used for myeloperoxidase activity measurement. Two experienced neuroradiologists blinded to the pathology results reviewed angiographic data to categorize the aneurysm morphology and measure the diameter of the aneurysm dome.

Statistical Analysis
The presence or absence of myeloperoxidase in each aneurysm based on histology score was binary coded. For each UIA, its 5-year aneurysm rupture risk (ARR) was determined using the Population, Hypertension, Age, Size of Aneurysm, Earlier Subarachnoid Hemorrhage From Another Aneurysm and Site of Aneurysm (PHASES) model. This model accounts for 6 risk factors including population (Finland, Japan, North American/other European countries), age, hypertension at baseline, prior history of subarachnoid hemorrhage, aneurysm size, and aneurysm location. The PHASES model does not account for sex, presence of multiple aneurysms, and smoking status at baseline because these additional factors did not add value to the model for predicting rupture. The data were statistically analyzed (SAS version 9.3, SAS Institute Inc., Cary, NC) to determine whether the distribution of ARR differed between the myeloperoxidase-positive and myeloperoxidase-negative groups. Logistic regression was used to determine the association between ARR and myeloperoxidase. We considered family history of subarachnoid hemorrhage, irregular aneurysm shape, and documented aneurysm growth as additional risk factors for rupture, and their association with myeloperoxidase was analyzed using Fisher exact test. Effects associated with P<0.05 were considered statistically significant.

Results
From January 2008 to July 2013, 23 aneurysms from 19 patients (13 women; median age, 54 years; age range, 29–75 years) were collected (Table I in the online-only Data Supplement). The average aneurysm diameter was 8.0±1.0 mm. Three were identified as ruptured, all of which were positive for myeloperoxidase. Of the 20 UIAs, half were positive for myeloperoxidase (Figure 1). Myeloperoxidase activity measurements in a small subset of aneurysms corroborated histological findings: In aneurysms stained negative for myeloperoxidase, there was 55.2±4.6 U myeloperoxidase/mg tissue (background level, n=2) that was ≈4-fold lower than the myeloperoxidase activity (200.5±17.8 U myeloperoxidase/mg tissue; n=4) in aneurysms found to be positive.

The ARR distribution did not satisfy the normality assumption (P<0.001, Shapiro–Wilk test). The median ARR for all UIAs was 1.4% and was higher for the myeloperoxidase-positive group (2.28%) than the myeloperoxidase-negative group (0.69%). Wilcoxon–Mann–Whitney test indicated that the distributions of 5-year ARR were statistically different between myeloperoxidase-positive and myeloperoxidase-negative groups (P<0.005). Logistic regression modeling ARR as a predictor of myeloperoxidase was statistically significant (likelihood ratio χ²: 8.44; P=0.004) and satisfied the goodness-of-fit Hosmer–Lemeshow test (P=0.506). The likelihood for myeloperoxidase-positive UIA was significantly associated (Wald χ²: 4.64; P=0.031) with ARR (odds ratio, 4.79; 95% confidence limits, 1.15–19.96).

Figure 1. Incidentally found 10-mm left middle cerebral artery (MCA) aneurysm in a 46-year-old woman (patient 17) is irregular with multiple blebs (A) and stains positive for myeloperoxidase (B; ×20). A 29-year-old woman (patient 16) with incidentally found right MCA and posterior communicating artery aneurysms on computed tomography angiography (C). The 14-mm MCA aneurysm stains negative for myeloperoxidase (D).
The receiver operating characteristic curve (Figure 3) had an area under the curve of 0.86. Positive myeloperoxidase staining also exhibited a statistically significant association with irregular aneurysm shape (Fisher exact test, \( P = 0.033 \), 2 sided) but not with the other risk factors considered (\( P > 0.068 \)). Addition of irregular aneurysm shape to the logistic regression model (with Firth bias correction) resulted in a statistically significant model (likelihood ratio \( \chi^2: 8.78; P = 0.012 \)) and improved the area under the curve (Figure 3).

Because clinically acceptable thresholds for PHASES estimated ARR have not been established, an ordered logistic regression model was used to determine whether myeloperoxidase was predictive of higher ARR by grouping the ARR in terms of the distribution quartiles (ARR \(<0.62\%: 0; 0.62\%\leq\text{ARR}<1.4\%: 1; 1.4\%\leq\text{ARR}<2.49\%: 2; \text{ARR}\geq2.49\%: 3 \)). The overall model was statistically significant (likelihood ratio \( \chi^2: 10.62; P = 0.001 \)), and the proportional odds assumption could not be rejected (\( \chi^2: 1.615; P = 0.446 \)). Myeloperoxidase was a significant predictor (\( P = 0.004 \)) of 5-year ARR ordered in terms of the distribution quartiles (proportional odds, 23.32; 95% confidence interval, 2.69–202.43).

Discussion

The role of neutrophilic myeloperoxidase as a potential contributing factor to UIA rupture has not been extensively studied, but a confluence of evidence from studies aimed at determining risk factors for atherosclerosis and other cardiovascular diseases identified myeloperoxidase as a key player in the progression of these diseases (reviewed in Schindhelm et al). More importantly, myeloperoxidase levels have been shown to be correlated with the severity and outcome of vascular disease, making it a highly promising biomarker for diagnosis and staging. In addition to its role in inflammation, myeloperoxidase mediates oxidative cell stress because of the production of reactive oxygen species. Chlorination of lipoproteins and nitrosylation of matrix proteins by myeloperoxidase are thought to contribute to vessel wall damage.

Inflammation is also an important element in the final stage of aneurysm development, that is, rupture, and the infiltration of neutrophils has been found to be greater in ruptured versus unruptured aneurysms. The emerging picture suggests that both aberrant vascular remodeling and inflammation contribute to aneurysm formation and progression, but that a key factor which may predispose the wall to rupture is ongoing inflammation mediated by an accumulation of neutrophils and monocytes/macrophages. Therefore, myeloperoxidase could play a critical role in aneurysm progression to rupture. Our analysis of myeloperoxidase in human UIAs indicates an association with risk of aneurysm rupture as predicted by the PHASES model, suggesting a potential role as a biomarker. It should be noted that our study design was limited by the requirement to obtain an aneurysm specimen at the time of surgery. We anticipate that with the future development of clinically acceptable noninvasive myeloperoxidase imaging probes, the patient selection bias of the current report will be overcome.

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Disclosures

None.

References

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**SUPPLEMENTAL MATERIAL**

**Supplemental Methods**

*Inclusion/Exclusion Criteria*

Inclusion criteria were diagnosis of a ruptured or unruptured brain aneurysm for which microsurgical clipping was performed in consenting patients between 18 and 79 years of age. Exclusion criteria included traumatic or mycotic aneurysms; aneurysms in patients with adult polycystic kidney disease; patients with concurrent degenerative connective tissue disorders; aneurysms associated with arteriovenous malformations; and patients with aneurysms that were not treated with surgical clipping as part of their standard of care during the hospitalization.

*Histology Protocol*

Tissue specimens were sectioned 8 µm thick, fixed with acetone, and blocked in 5% bovine serum, 10 mg/ml BSA in 50 mM Tris, 100 mM NaCl (TBS), pH 7.5 for 2 h. The staining of human MPO and macrophage (anti-calprotectin) antigens was performed using the corresponding mouse anti-human monoclonal antibodies (2C7 and MAC387, dilution: 1:100, AbCam, Cambridge MA). Mouse monoclonal antibody binding was detected using anti-mouse monoclonal alkaline phosphatase-conjugated secondary antibody staining with subsequent NBT/BCIP colorimetric detection (Roche Applied Science, Indianapolis IN). Sections were counter-stained with nuclear fast red. Three non-consecutive sections were examined using light microscopy. The collected RGB JPEG images (magnification: 30x) were color split and the corresponding blue channel images were segmented using IP Lab Spectrum software (BD Biosciences Bioimaging, Rockville MD). The MPO staining was considered positive if > 5 alkaline-phosphatase positive cells were present in at least one field with an area of 400 µm². Representative images are shown in Supplemental Figure I.

When the aneurysm specimens were sufficiently large, they were carefully cut in half along the long axis of the dome for both histology and MPO activity analysis. The analysis of MPO activity
was performed by using a commercially available MPO kit (Fluoro MPO, Cell Technology Inc., Mountain View CA). A typical assay included homogenization of 1-10 mg of saline-rinsed, rapidly defrosted tissue in a vial containing a slurry of 300 mg sterile glass beads (1mm diameter) suspended in 0.5 ml of 0.5% solution of hexadecyltrimethylammonium bromide, 10mM N-ethylmaleimide in 0.1 M potassium phosphate, pH 6.5. The homogenization was performed using a mini-BeadBeater (BioSpec Products, Inc. Bartlesville OK) 12 cycles (30 s each) with 1 min cooling on ice in between the cycles. The final disruption was performed by using three freeze-thaw cycles. The samples were cleared by centrifugation (8000xg, 5min) and the activity of MPO was determined in the supernatant using a fluorescent analog of MPO substrate in the presence of hydrogen peroxide. The rates of fluorescence increase was determined using a kinetic assay ($\lambda_{ex}=550$ nm, $\lambda_{em}=600$nm) of an MPO standard solution (Cell Technology Inc.) to generate calibration curves. The protein content in the homogenized samples was determined by using BCA kit (Bio-Rad Inc, Hercules CA).

Supplemental Figure

Supplemental Figure I. A- a representative MPO- negative field, B- an MPO-positive field. Bar=100 µm
### Supplemental Results

#### Supplemental Table I – Results of human aneurysm tissue histology

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Abbreviations: D = diameter, SAH = subarachnoid hemorrhage, HTN = hypertension, MPO=myeloperoxidase, ARR=5-year aneurysm rupture risk (for unruptured aneurysms) estimated using PHASES model, ICA=internal carotid artery, ACA=anterior cerebral artery, AComm=anterior communicating artery, MCA=middle cerebral artery, PComm=posterior communicating artery, Unk=unknown, ATA=anterior temporal artery; Unk=Unkown; * -
indicates documented aneurysm growth during observation; ** indicates patient had prior history of SAH.